

## REMARKS

Upon entry of the present amendment, claims 1-3, 5, 6 and 9-12 are pending. Claims 1, 2 and 12 have been amended to replace the term “a coding sequence” of SEQ ID NO:2 with “the coding sequence” of SEQ ID NO:2. No other claim amendments have been presented herein. Accordingly, no new matter has been added by this amendment.

### **Rejections Under 35 U.S.C. §112, second paragraph**

The Examiner has rejected claim 1 under 35 U.S.C. § 112, second paragraph as indefinite for failing to distinctly claim the coding sequences of SEQ ID NO:2. According to the Examiner, the term “a coding sequence of SEQ ID NO:2 implies there are multiple coding regions of SEQ ID NO:2 which from the specification as filed the sequence is a human cDNA and by character can only have a single coding region.” (Office Action, page 2).

Claims 1, 2 and 12 have been amended to replace the term “a coding sequence” of SEQ ID NO:2 with “the coding sequence” of SEQ ID NO:2. Accordingly, all references to “a coding sequence” of SEQ ID NO:2 have been removed from the pending claims. The specification provides the nucleic acid sequence of SEQ ID NO:2 in conjunction with the amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO:2, *i.e.*, the amino acid sequence of SEQ ID NO:4. In light of this disclosure, the skilled artisan in the field of molecular biology will readily appreciate the scope and meaning of the claim term “the coding sequence of SEQ ID NO:2”. Accordingly, Applicants submit that amended claims 1, 2 and 12 distinctly claim the coding sequence of SEQ ID NO:2, and this rejection should be withdrawn.

### **Rejections Under 35 U.S.C. § 112, first paragraph**

Claims 1-3, 5, 6 and 9-12 have been rejected under 35 U.S.C. §112, first paragraph for lack of enablement. According to the Examiner, the specification does not teach:

(1) stability of the antisense molecule *in vivo*, (2) delivery to the whole organism and specificity to the target tissues, (3) dosage and toxicity, nor (4) entry of the molecule into cell and effective action therein marked by visualization of the desired treatment effects. There is no guidance in the specification as filed that teaches how the claimed antisense compounds enter the human macrophage cell, inhibit the expression of AFABP, prevent the human macrophage from differentiating into a foam cell and ultimately inhibit atherosclerotic lesions. (Office Action, pages 3-4).

Section 112, first paragraph, requires that the specification provide a description that, when coupled with the knowledge possessed by a person of ordinary skill in the art, enables that person to make and use the claimed invention. Atlas Powder Co. v. E.I. duPont De Nemours & Co., 750 F.2d 1569, 1576 (Fed. Cir. 1984). Enablement is not precluded by the necessity for some experimentation; however, any required experimentation must not be undue experimentation. In re Wands, 858 F.2d 731, 736-7 (Fed. Cir. 1988).

In re Wands sets forth eight factors to be analyzed in determining whether undue experimentation is required to practice the claimed invention: (A) the breadth of the claims; (B) the nature of the invention; (C) the state of the prior art; (D) the level of one of ordinary skill; (E) the level of predictability in the art; (F) the amount of direction provided by the inventor; (G) the existence of working examples; and (H) the quantity of experimentation needed to make or use the invention based on the content of the disclosure. MPEP at 2164.01 (a).

Applicants submit that, in weighing all eight factors, the specification meets the standard for enablement for the full scope of the claimed invention.

#### Breadth of the claims

The amended claims are directed to methods of inhibiting formation of an atherosclerotic lesion by contacting a macrophage of a mammal with a compound that reduces expression of AFABP, wherein (i) AFABP includes the amino acid sequence of SEQ ID NO:4, (ii) a reduction in AFABP expression inhibits formation of an atherosclerotic lesion, and (iii) the compound is an nucleic acid comprising 10-100 nucleotides, the sequence of the nucleotides being complementary to at least 10-100 nucleotides of the coding sequence of SEQ ID NO:2.

Thus, the pending claims are not directed to methods of inhibiting formation of an atherosclerotic lesion by contacting any cell with any compound that reduces the expression of AFABP. Rather, the amended claims are directed to a particular subset of compounds that reduce expression of AFABP, namely antisense oligonucleotides that include 10-100 nucleotides having a nucleic acid sequence that is complementary to at least 10-100 nucleotides of the coding sequence of SEQ ID NO:2. In addition, the claimed methods require the use of a specific type of cell - a macrophage.

Accordingly, Applicants submit that the amended claims are not overly broad, particularly in light of the contribution of the claimed invention to the field of cardiovascular medicine. As described throughout the as-filed specification (e.g., at page 11, lines 1-2), “prior

to the invention, it was thought that AFABP expression was limited to adipocytes.” Applicants made a significant contribution to the field of cardiovascular medicine by showing that “AFABP was found to be expressed in macrophages and macrophage-derived foam cells associated with atherosclerotic lesions (but not circulating monocytes).” (*See e.g.*, specification at page 11, lines 3-5). Therefore, Applicants submit that the claims are commensurate with their contribution to the field.

Nature of the invention and State of the prior art

These factors are not explained in Wands, but Applicants believe that it is reasonable to assume that the court was referring to the foundation in the art for the claims and the advance represented by the claims. The nature of the invention is inhibition of atherosclerotic lesions.

As described above, Applicants have made an important contribution to the field of cardiovascular medicine by demonstrating that AFABP is expressed in macrophages and macrophage-derived foam cells associated with atherosclerotic lesions. Exploitation of this discovery according to the amended claims allows specific targeted inhibition of atherosclerotic lesion development by inhibiting macrophages from differentiating into foam cells. Thus, the claimed methods represent a significant advance in the field of cardiovascular medicine.

Level of one of ordinary skill in art

Applicants submit that the level of skill of those skilled in the field of medicine concerned with atherosclerosis and in the field of gene therapy is very high. Many years of post-graduate training and experience is required. Moreover, skilled artisans have been practicing in the field atherosclerosis and inhibition thereof (including gene therapy and antisense approaches) for many years. Accordingly, armed with the information provided in the specification regarding which compounds to administer, how to administer the compound, and what kind of cells to contact, those skilled in the art would readily be able to carry out the invention as now claimed. Undue experimentation would not be required for one skilled in the art to carry out the invention recited by the amended claims.

Predictability or unpredictability of the art

With respect to the this factor, the Examiner stated that unpredictability is known in the antisense art for therapeutic, *in vivo* applications. According to the Examiner, “it is not predictable that an antisense which inhibits a target gene in cells in culture will function

equivalently in a whole organism in view of the numerous unpredictable considerations found in whole organisms.” (Office Action, page 4). The Examiner has cited to the Crooke reference (Crooke, *Currently Molecular Medicine*, vol. 4(5):465-487 (2004) at page 471, col. 1) as indicating that “clear demonstration of the antisense mechanisms are required before drawing conclusions from *in vitro* experiments”.

Applicants’ respectfully disagree with the Examiner’s characterization of the field of antisense technology, particularly in the instant case, where the claimed antisense oligonucleotides are limited to a specific gene target. Although antisense methods may require further experimentation to optimize the desired result of reduced expression of a target gene, the methodology is well established and well accepted by the scientific and medical community. Numerous antisense compositions are currently being administered to human subjects and antisense technology is regarded as a sound therapeutic approach. A discussion of the antisense oligonucleotides that are undergoing/have undergone clinical trials is presented in the attached copy of a review by Dr. Richard Hogrefe, entitled “An Antisense Oligonucleotide Primer”

In fact, Dr. Hogrefe notes that with regard to the *in vivo* delivery of therapeutic oligonucleotides:

Perhaps one of the most surprising findings to come out in the last 15 years in this field is that while delivery into cells *in vitro* appears to be difficult, *in vivo* delivery does not appear to be a problem. That statement is very bold and simplistic - and even controversial - but is well supported since all of the oligonucleotides in clinical trials are administered as saline solutions without delivery vehicles. (page 1, col. 2)

Accordingly, Applicants submit that the field of *in vivo* antisense applications is not “highly unpredictable”. Once a target gene is identified and validated, developing a therapeutic construct is well within the scope of predictability encountered by a skilled artisan working in the field of clinical drug development and within the scope of reasonable experimentation permitted by the enablement standard.

Amount of direction or guidance presented

The greater the amount of guidance provided, the more this factor weights in favor of granting the claim.

The specification of the present application provides ample guidance regarding the procedures required to carry out the methods of the invention. For example, the specification provides ample examples of methods that will identify compounds which bind to at least 10-100

nucleotides of SEQ ID NO:2 and inhibit expression of AFABP, *e.g.*, as described on page 14, line 29 through page 17, line 9. These screening methods include cell-based screening methods, such as, for example, methods that use macrophages and detect AFABP expression in the presence of a test antisense oligonucleotide, as well as methods that detect the effect of a test compound on macrophage differentiation into foam cells. Thus, the use of these cell-based techniques to identify which molecules inhibit AFABP expression also simultaneously identifies those molecules that are able to enter a human macrophage cell. Techniques for optimizing the method of delivering the test compounds are routine in the art and would not require undue experimentation for the skilled artisan.

In addition to these methods of identifying compounds that inhibit the expression of AFABP, the specification also provides art-recognized methods and related references of administering the identified compounds to a subject (*see e.g.*, page 9, line 8 through page 10, line 12). Thus, Applicants submit that adequate guidance is provided in the specification of the application to allow one skilled in the art to identify compounds that inhibit the expression of AFABP and to carry out the claimed invention without having to resort to “trial and error experimentation”.

#### Presence or absence of working examples

The working examples provided in the as-filed specification were conducted in the art-recognized mouse model of vascular disease. As acknowledged by the Examiner, the evidence provided in this working examples clearly demonstrate that there is a correlation between decreased levels of AFABP and decreased atherosclerotic lesions. Accordingly, these examples provide evidence that compounds encompassed by the claims, namely those that reduce the expression of AFABP, inhibit the development of atherosclerotic lesion in mammalian tissue.

#### Quantity of Experimentation Necessary

In *Wands*, the Court stated,

[A] considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

Applying this criterion here, all of the techniques required to practice the claimed methods were described in the specification or were well-known to those skilled in the art as of

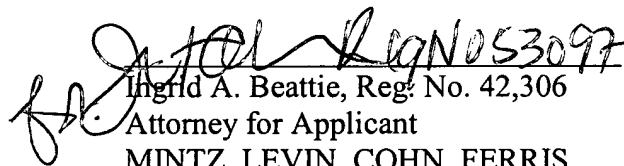
the filing date. Little or no experimentation is required to determine which compounds to administer. As described in the specification (*e.g.*, page 9, lines 25-29), "oligonucleotides that are complementary to a portion or all of the AFABP mRNA are readily tested *in vitro* for their ability to decrease production of AFABP" using the assays described on page 14, line 29 through page 17, line 9. Since extensive guidance is provided regarding modes of administration, minimal experimentation would be required to administer the compounds. Although some experimentation may be required to determine the concentration or dose of the compound to be administered, such determinations are routine in the art and would not require undue experimentation.

Accordingly, Applicants submit that, in weighing all eight factors, the specification meets the standard for enablement for the full scope of the claimed invention. The Examiner should, therefore, withdraw this rejection.

### CONCLUSION

Applicants submit that the application is in condition for allowance and such action is respectfully requested. Should any questions or issues arise concerning the application, the Examiner is encouraged to contact the undersigned at the telephone number provided below.

Respectfully submitted,

  
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Attachments:

-- APPENDIX: "An Antisense Oligonucleotide Primer" by Richard Hogrefe, Ph.D.;  
TriLink BioTechnologies (6 pages)

# An Antisense Oligonucleotide Primer

By Richard Hogrefe, Ph.D.; TriLink BioTechnologies

(an adaptation of this article was published in *Antisense and Nucleic Acid Drug Development*, 9, 351-357 1999. Table 1 updated 2002.)

Antisense oligonucleotides are short, synthetic strands of DNA (or analogs) that are complimentary, or antisense, to a target sequence (DNA or RNA) designed to halt a biological event, such as transcription, translation or splicing. After a period of doubt (Gura, 1995; Stein, 1995), antisense has been resurrected as a powerful tool for the molecular biologist and the first antisense drug (Isis's Fomivirsen) recently received FDA approval. The antisense field is experiencing an explosion of interest now that the phenomenon of the inhibition of gene expression by antisense oligonucleotides is more or less universally acknowledged. This essay will hopefully help the novice understand a few key principles regarding the use of antisense technology, as well as learn ways to avoid costly errors that nearly caused the premature death of the field.

Paul Zamecnik is generally attributed with publishing the first paper describing the use of antisense oligonucleotides (Stephenson, 1978). The early theory held by many was that if a synthetic oligonucleotide was annealed to a single stranded mRNA (or genetic DNA), the ribosome (or polymerase) would not continue reading the code and fall off, thus effecting 'hybrid arrest'. In fact, it was later learned that ribosomes and polymerases are indeed more like trains and will quickly read through the hybridized region. The true mechanism of action, in fact, is enzymatic cleavage of the RNA strand by RNase H (Minshull and Hunt, 1986; Dash, *et al.* 1987; Walder and Walder, 1988).

Even before the role of RNase H was discovered, it became apparent that, in order to develop an antisense drug, one of the hurdles that needed to be overcome was the rapid degradation of the oligonucleotide in the blood and in cells by both exonucleases and endonucleases. To remedy this, modified backbones were introduced that resisted nuclease degradation (Blake, *et al.*, 1985; Agrawal and Goodchild, 1987). These modifications included subtle as well as not too subtle changes to either the phosphate or the sugar portion of the oligonucleotide. As it turned out, phosphorothioates, one of the easiest modifications to synthesize, have been the most successful to date (Matsukura, *et al.*, 1987; Stein, *et al.*, 1988).

One of the most simple and straightforward modifications that can be made to an oligonucleotide is to replace a non-bridging oxygen on the phosphate backbone with sulfur, producing a phosphorothioate linkage. The ability of this modification to retard nuclease degradation of oligonucleotides was long known (Matzura and Eckstein, 1968). It was later learned that this modification is also a substrate for RNase H (Stein, *et al.*, 1988; Furdon, *et al.*, 1989). These properties, combined with the relative ease of synthesis, have led to the ascendancy of this compound as an antisense drug. However, the road has not been easy. It was rapidly discovered that these compounds exhibited several unexpected properties *in vivo* (Srinivasan and Iversen, 1995). Despite the issues, most of the compounds progressing through clinical trials at this time are phosphorothioates (Table 1).

## Target Validation versus Antisense Drug Development

The design of your experiments is predicated on where your interest in antisense lies. Of the many potential applications of antisense, two are most prominent: target validation and the use of an oligonucleotide as an actual therapeutic agent. Target validation refers to the use of antisense oligonucleotides in cell culture to determine if down regulating a certain gene target will give desired biological results (i.e. tumor cell line reduction, etc.). This information is often used

to help develop more classic small molecule drugs.

Fortunately, much of the early phase work needed to develop a therapeutic agent is similar to what is needed to conduct target validation experiments, which is why so many antisense drug companies are now antisense target validation companies as well. However, several significant differences do exist. Those interested in using antisense as a tool to study genes will most likely work exclusively with cells, which requires less material, but more attention to the issue of cellular uptake. Those interested in developing an antisense drug must concern themselves with scale up, toxicity, delivery, pharmacokinetics, and the FDA, of course.

One very important difference in developing an *in vitro* assay or a therapeutic drug is choice of oligonucleotide construct. When designing an antisense drug, one of the overriding concerns needs to be the ability to scale up the synthesis of the construct for a reasonable cost. This is the reason that most of the constructs in clinical trials are phosphorothioate oligonucleotides with no other modification. They are the least expensive of the constructs that have the correct properties for an antisense drug - nuclease resistance and retention of RNase H activity. However, there are problems with phosphorothioates that will be discussed in more detail below. When designing an antisense *in vitro* assay for target validation you have more flexibility and can choose from the newer "second generation" constructs that will also be discussed below.

The other major difference between *in vivo* and *in vitro* work is cellular uptake and delivery. Perhaps one of the most surprising findings to come out in the last 15 years in this field is that while delivery into cells *in vitro* appears to be difficult, *in vivo* delivery does not appear to be a problem. That statement is very bold and simplistic - and even controversial - but is well supported since all of the oligonucleotides in clinical trials are administered as saline solutions without delivery vehicles. The whole field of antisense drug development almost collapsed when it was determined that a delivery system was needed in order to be successful *in vitro*. The potential cost of developing such a vehicle for systemic delivery of oligonucleotides *in vivo* frightened an investment community that already spent a great deal of money and was losing patience. Fortunately, a brave few went ahead and injected animals anyway only to discover that in some instances results were obtained that were convincingly due to an antisense mechanism (Dean and McKay 1994; Desjardins and Iversen, 1995). The occasional positive therapeutic effects that cannot be proved to be antisense should be considered an added bonus (Stein, 1995; Ramasamy, *et al.*, 1996; Boiziau, *et al.*, 1997). In any case, regardless of whether you are developing an antisense drug or an *in vitro* assay, your initial studies will most likely be with cells in culture and therefore you will have to be concerned with uptake.

## Choosing an Oligonucleotide Construct

As stated earlier, the choice of backbone construct depends on your final goal. If you are designing an antisense drug, you must consider the cost to synthesize the final product and if it is even feasible in large scale. The chemistry must precede the biology to a large extent. This should be a primary concern. If you have novel chemistry, or very complicated chemistry, seek the counsel of someone experienced with oligonucleotide synthesis from a commercial and scale up perspective. Make sure that others can reproduce your work.

# The Art of Antisense

**Table 1: Oligonucleotides in Clinical Trials**

Name of Oligo	Sponsor	Sequence	Chemistry	Target	Disease	Route of Administration	Status (Phase)
Vitravene™	Isis/Novartis	GCGTTTGCTCTTCTT CTTGCG	Phosphorothioate	IE2	CMV Retinitis	Intravitreal	On Market
Affintak™	Isis/Lilly	GTTCTCGCTGGTGAGTTCA	Phosphorothioate	PKC-α	Cancer - NSCLC, others	Parenteral	III
Allcaforsen™	Isis	GCCCAAGCTGGCATCCGTCA	Phosphorothioate	ICAM-1	Crohn's Disease	Parenteral	III
ISIS 2302	Isis	GCCCAAGCTGGCATCCGTCA	Phosphorothioate	ICAM-1	Topical Psoriasis	Topical	II
ISIS 2302	Isis	GCCCAAGCTGGCATCCGTCA	Phosphorothioate	ICAM-1	Ulcerative Colitis	Enema	II
ISIS 2503	Isis	TCCGTCATCGTCTCAGGG	Phosphorothioate	H-ras	Cancer - pancreatic, others	Parenteral	II
ISIS 14803	Isis/Elan	GTGCTCATGGTGACGGTCT	Phosphorothioate (all Cs are 5-methyl-dC)	Antiviral	Hepatitis C	Parenteral	II
ISIS 104838	Isis/Elan	GCTGATTAGAGAGGTCCC	Chimeric (deoxy gapped) Phosphorothioate	TNF-α	Rheumatoid Arthritis	Parenteral/Oral	II
ISIS 104838	Isis	GCTGATTAGAGAGGTCCC	Chimeric (deoxy gapped) Phosphorothioate	TNF-α	Psoriasis	Topical	II
OGX-011	Isis/Oncogenex	N/A	N/A	Clusterin	Cancer	Parenteral	I
Genasense™ (G3139)	Genta	TCTCCCAGCTGCGCCAT	Phosphorothioate	Bcl-2	Cancer	Intravenous	II/III
E2F Decoy	Corgentech	N/A	N/A	E2F	Artherosclerosis	Ex-vivo	II/III
AP 12009	Antisense Pharma	N/A	Phosphorothioate	TGFβ2	Glioma	Intra-tumoral	I/II
GTI-2040	Lorus	N/A	Phosphorothioate	RNR	Cancer	Intravenous	II
GTI-2501	Lorus	N/A	Phosphorothioate	RNR	Cancer	Intravenous	I
GEM*231	Hybridon	<u>GCGUGCCTCCTCACUGGC</u>	Chimeric Phosphorothioate	PKA	Refractory Solid Tumors	Intravenous	I/II
MG98	Methyligene/Hybridon	N/A	Mixed backbone	DNA MeTase	Lung, colon and breast cancer	Intravenous	I
EPI-2010	Epigenesis	GATGGAGGGCGGCATGGCGGG	Phosphorothioate	Adenosine A <sub>1</sub> Receptor	Asthma	Aerosol	II
MBI 1121	Micrologix	N/A	N/A	E1 region of HPV	Genital warts	Topical	I
<b>Notes:</b> The underlined bases in GEM-231 are 2'OMe sugar modifications. N/A: Not Available or Company will not disclose. Table continued on next page.							



# The Art of Antisense

**Table 1: Oligonucleotides in Clinical Trials, continued**

Name of Oligo	Sponsor	Sequence	Chemistry	Target	Disease	Route of Administration	Status (Phase)
Resten-NG	AVI BioPharma	N/A	NeuGene (Morpholino)	c-myc	Restenosis	Intravenous	III
Oncomyc-NG	AVI BioPharma	N/A	NeuGene (Morpholino)	c-myc	Cancer	Intravenous	II
AVI-4014	AVI BioPharma	N/A	NeuGene (Morpholino)	NFκB	Inflammation	Intravenous	I
AVI-4126	AVI BioPharma	N/A	NeuGene (Morpholino)	c-myc	PKD	Intravenous	II
AVI-4557	AVI BioPharma	N/A	NeuGene (Morpholino)	cyp3a4	Cancer/ Other diseases	Intravenous	II
ProMune™	Coley Pharma	N/A	CpG motif B-Class Oligo	Immune Stimulant	Many	Intravenous	I/II
HEPTAZYME™	RPI	Ribozyme	RNA/DNA	HCV	Hepatitis C	Intravenous	II
ANGIOZYME™	RPI	Ribozyme	RNA/DNA	VEGFR-1	Breast Cancer	Intravenous	I/II
ANGIOZYME™	RPI	Ribozyme	RNA/DNA	VEGFR-1	Colorectal Cancer	Intravenous	I/II
HERZYME™	RPI	Ribozyme	RNA/DNA	HER2	Cancer	Intravenous	I
R-95288	Sankyo KK	DMT-TGGGAG	Aptamer, Phosphodiester DNA	HIV-1	AIDS	Intravenous	I
HGTV43	Enzo	Stealth Vector™ (gene transfer vector)	DNA	T-cells	HIV/AIDS	Ex-vivo	I
Product R	Advanced Viral Peptide Nucleic Acid Research Corp.		PNA	CCR5	HIV	Unknown	I/II
1018-4SS	Dynavax	N/A	ISS Technology (Phosphorothioate)	Immune Response	Hepatitis B	Intravenous	II/III
1018-4SS	Dynavax	N/A	ISS Technology (Phosphorothioate)	Immune Response	Ragweed Allergy	Intravenous	II/III
1018-4SS	Dynavax	N/A	ISS Technology (Phosphorothioate)	Immune Response	Asthma	Intravenous	I/II
1018-4SS	Dynavax	N/A	ISS Technology (Phosphorothioate)	Immune Response	NHL	Intravenous	I/II
<p><b>Adapted from:</b> Sanghvi, Y.S. <i>et al.</i> in <i>Manuals of Antisense Methodology</i>. Eds., Hartmann, G., and Endres, S., Kluwer Academic Publisher, 1998. Updated in January 2003 by TriLink. This table was compiled to the best of our ability, however there may be additional compounds that are currently under clinical trials.</p> <p><b>N/A:</b> Not Available or Company will not disclose.</p>							

The most popular modification for antisense oligonucleotides continues to be phosphorothioates. These oligonucleotides can be obtained for fairly reasonable prices and in kilogram scales. Antisense firms still commit a great deal of their development budget to improving the synthesis of this compound. They obviously expect a continual stream of phosphorothioate oligonucleotides to enter the clinic, as well as obtain FDA approval. Phosphorothioate oligonucleotides are probably a good choice if you wish to rapidly develop a program. Besides price, phosphorothioate oligonucleotides have demonstrated success in a science where success has often been difficult to achieve.

However, there are some problems with phosphorothioate oligonucleotides. The backbone is chiral, resulting in a racemic mixture of 2' oligonucleotide species (where n = number of phosphorothioate internucleotide linkages) instead of a single compound. The overall mixture has a lower  $T_m$  than its corresponding phosphodiester oligonucleotide (LaPlanche, *et al.*, 1986). Phosphorothioate oligonucleotides have been known to exhibit unusual properties *in vivo*, both desirable and undesirable. Some of those effects are due to the affinity phosphorothioates show for proteins (Brown, *et al.*, 1994). Phosphorothioates also have a reputation for being toxic (Srinivasan and Iversen, 1995), although that may be a sequence specific phenomenon or due to contamination in early oligonucleotide preparations. Another problem for some is that the NIH patented phosphorothioate oligonucleotides for antisense applications. Be prepared to pay Uncle Sam for the right to use this compound as a therapeutic agent once you succeed.

For those who want to have alternatives, or wish to develop an antisense *in vitro* assay as an endpoint, other possibilities do exist. These "second generation" oligonucleotide constructs are available commercially and the less complex ones are not much more expensive than phosphorothioate oligonucleotides at the smaller scales. In fact, most of them include some phosphorothioate linkages, and many are still completely modified with phosphorothioates. A common design is to have nuclease resistant arms (such as 2'-O-methyl (OMe) nucleosides) that surround a phosphorothioate modified deoxyribose core that retains the RNase H activity of the oligonucleotide (Agrawal and Goodchild, 1987; Giles and Tidde, 1992). Oligonucleotides that contain mixtures of chemistry are called chimeric oligonucleotides. Chimeric oligonucleotides containing 2'-OMe arms were used to help understand the underlying principles of the RNase H mechanism (Hogrefe, *et al.*, 1990). The most significant enhancements offered by this class of compound are a general reduction in toxicity, increased hybrid stability, and increased nuclease stability (Peng Ho, *et al.*, 1998; Zhou and Agrawal, 1998). These all combine to yield a compound more reproducibly active when used in an *in vitro* assay and are your best choice for such work.

Since 1987, various groups have staked out several specific constructs as proprietary. In the end you may have to seek counsel of a good patent attorney and obtain a license for a particular construct, but for research purposes a number of choices are available commercially. A good starting point is to use an oligonucleotide eighteen nucleotides in length that has six 2'-OMe nucleotides at both the 5' and 3' ends, leaving a core of six 2'-deoxyribose nucleosides with phosphorothioate internucleotide linkages (Monia, *et al.*, 1993; Metele, *et al.*, 1994). The arms may or may not contain phosphorothioate linkages for best results. Removing phosphorothioate linkages may reduce toxicity, however it also reduces nuclease resistance. You have to see what works best with your system.

"Third generation" compounds are also in development. These constructs are a return to the original concept of

hybrid arrest and depend on extreme hybridization enhancement using highly modified oligonucleotides. These modifications include 2'-MOEs (Monia, 1997), N3'-P5' phosphoramidates (Gryaznov and Chen, 1994; Mignet and Gryaznov, 1998), PNA's (Hanvey, *et al.*, 1992), chirally pure methylphosphonates (Reynolds *et al.*, 1996), MMIs (Morvan, *et al.*, 1996; Swayze, 1997), and others. While most of these constructs work to some extent, all have at least one significant problem, such as solubility, delivery, or cost of synthesis. At this time, it is probably best not to explore these types of compounds unless you have extensive in-house experience.

As a final word regarding the oligonucleotide itself, whatever the construct you choose, be certain of the integrity of the compound. Many of the failed experiments and false conclusions of the past were due to contamination. Although there has been significant improvement over the years, there is still a need to be attentive to purity, particularly as the modification requirements increase.

### Choice of Sequence

There is no sure way to determine *a priori* where on a particular gene is the most active site for an antisense oligonucleotide, although advice does exist (Cohen, 1989; Woolf, *et al.*, 1992; Brysch and Schlingensiepen, 1994). The region surrounding the start codon (AUG) site is probably the most popular, followed by site mutations. Recently, targeting splicing sites has become increasingly popular in order to inhibit the mRNA processing mechanism as opposed to the message (Sierakowaka, *et al.*, 1996).

For every site of interest, design up to ten different sequences along the region, trying to maximize hybridization while avoiding sequences with regions of polyguanosine or G-C arms that will form strong hairpins. There is a good chance that one of those ten sequences will be active. Some of the chimeric oligonucleotides have even better success rates.

You do have to be concerned with what is commonly referred to as the CpG effect. Some oligonucleotide sequences that contain the dinucleotide CpG cause a fairly profound stimulation of the immune system (Krieg, 1998). The explanation is that in mammalian cells, most of the exposed cytosine is methylated at the 5 position. Bacterial cytosine is not methylated. Apparently, mammals have developed an immune response to non-methylated genomic material as a defense against bacteria. Several groups are actually exploiting this effect for its therapeutic value (Klinman, 1998; Millan, *et al.*, 1998). If you are concerned about the effect a CpG may have on your system, a simple experiment is to replace all the cytidines 5' to guanosines with 5-methylcytidine which will inhibit the effect (Boggs, 1997).

### Developing a Successful *In Vitro* Experiment

Whether you are developing an antisense drug or an *in vitro* assay, the initial experiments will most likely be with cells in culture. This, of course, is your endpoint when developing an *in vitro* assay, but it is also highly recommended when developing an antisense drug. In general, it is easier to look for true antisense indicators such as reduction of target mRNA or protein *in vitro* than *in vivo*. The screening process is also far more economical. What must be remembered is that to be successful *in vitro*, you must use a delivery system. Conversely, you can go forward with your *in vivo* experiments sans carrier once you've discovered a good target. There does not appear to be a satisfactory explanation for this phenomenon.

Fortunately, we do seem to have a reasonable solution to the problem of *in vitro* cellular uptake. The most effective delivery system has turned out to be cationic lipids (Capaccioli, *et al.*, 1993; Lappalainen, *et al.*, 1994; Quattrone, *et al.*, 1995), which have become the standard for *in vitro* work.

## The Art of Antisense

The one caveat is that there is no universal cationic lipid that works for all cell lines and with all constructs. Worse yet, sometimes a mixture of different cationic lipids at specific concentrations is required to optimize delivery into your particular cell line. The plain fact is that you have to hand tailor the cationic lipid mixture to fit your cell line and oligonucleotide construct.

Many commercially available cationic lipids exist. It is highly likely that amongst them at least one uptake system can be found that works. However, if you are exploring your own cell line or oligonucleotide construct, then you are going to have to do some experimentation to find the right delivery system. To make it easier, kits are available that contain various lipid mixtures for this expressed purpose.

The best way to begin developing a successful *in vitro* assay is to determine conclusively if good cellular uptake is occurring with your delivery system by using a fluorescently labeled oligonucleotide and fluorescence microscopy to observe uptake (Noonberg, *et al.*, 1992; Sasaski, *et al.*, 1995). The sequence is relatively unimportant here. All that matters is that the construction is the same as what you intend to use. Although a fluorescent molecule must be added, this does not appear to affect uptake. Since most fluorescent microscopes come equipped with filters for fluorescein, that fluorophore will serve well. Please note that the fluorescent molecule must be introduced during the synthesis of the oligonucleotide. Therefore a separate preparation of your compound is required. As an alternative, inexpensive fluorescently labeled oligonucleotides with mixed base compositions are available for this purpose from commercial sources.

The experiment to study the uptake of your fluorescently labeled oligonucleotide is fairly straightforward (Shoji, *et al.*, 1991; Sasaki, *et al.*, 1995). The fluorescently labeled oligonucleotide is mixed with the cationic lipid mixture(s) according to the manufacturer's instructions. These are applied to the cells as 1 to 3 micromolar solutions. The cells are harvested and fixed, then viewed under the microscope. The desired effect is uptake into the nucleus of the cells. A fair proportion of the cells should have fluorescence in the nuclei. This is indicative of proper delivery. If a punctate pattern (isolated spots of fluorescence) is visible in the cytoplasm instead, then you are merely observing endoplasmic sequestering, which was the fate of most of the oligonucleotides used in the early experiments without delivery systems (Shoji, *et al.*, 1991).

Once you have found a satisfactory lipid mixture, you can test your antisense sequences with the confidence that you are truly looking at activity. It is actually a good idea to periodically test your delivery system using a fluorescent oligonucleotide to make sure that the cell line hasn't transformed in some manner that changes uptake properties.

One last question to answer in regard to *in vitro* experimentation is how much oligonucleotide is required. Usually 5 to 10 ODs (~150 - 300 µg) which is readily obtained from a 200 nmole scale synthesis is more than enough. For more extensive experimentation, a 1 µmole scale synthesis will generally yield 1 to 2 mg of purified oligonucleotide, depending on the construct. The extra material will also allow you to retain sample to compare with new batches of oligonucleotide in case activity changes, which is not uncommon.

### Moving to *In Vivo* Experiments

When you start your *in vivo* studies you have a whole new set of concerns. First, you must be assured of obtaining a reasonable quantity of your oligonucleotide. Initially, your requirements will not be extensive. A fairly comprehensive rodent study can be conducted with 50 mg of oligonucleotide. At a common dose of 5 mg/kg, 500 inoculations can be made to mice that normally weigh 0.02 kg. However, a rat study will obviously go through 50 mg much more rapidly. Later experiments will require grams of material.

If you are using standard phosphorothioate oligonucleotides, or one of the more common chimeric oligonucleotides, supply will not be a significant concern. However, if your construct is fairly complicated, be sure to investigate scale up issues early in the program, rather than later. Nothing is worse than spending millions developing a drug only to find out it is next to impossible to manufacture for a reasonable fee.

Along with quantity goes quality. It is even more important to be sure of the quality of your material. Toxicity due to contaminants is very easy to avoid if proper precautions are taken. Be picky and willing to pay good money for good material, whether it is from an in-house source or from an external vendor.

A very significant concern is how to interpret the results of your experiments and prove you have an antisense drug. This has been a topic of controversy from the beginning. It is difficult to locate and quantitate the reduction of both target mRNA and protein product. Still, it has been done (Monia, 1997) and is the best way to be confident in an antisense mechanism. One strong argument that is very persuasive is championed by adherents to the "So what?" school of thought. If the compound does what it was meant to do, why argue? The practical course is to accept positive results and continue towards a drug product. You can always continue the search for the mechanism of action later with the hope of discovering the Holy Grail of drug development - true rational drug design.

Another concern, or perhaps a relief, is that you can throw out the delivery system you so arduously developed for your cell work. Most *in vivo* oligonucleotide solutions are merely saline. The solutions are injected in various ways, including ocular and other locations even more difficult to imagine enduring. Oral delivery has also been examined (Agrawal, *et al.*, 1995). Despite the apparent success of some ongoing trials using no delivery system, there is still a fairly universal belief that a good method of systemic delivery to specified tissues can only be advantageous and lead to a higher success rate. The merging needs, in fact, have led to merging companies, such as the purchase of Lynx's therapeutic oligonucleotide technology by Inex of Canada, a delivery company. Until such a delivery system emerges, your best course is to just use a saline solution.

Another set of experiments unique to the development of an antisense therapeutic is the need for pharmacokinetic studies. These animal experiments require injection of the test subject with an oligonucleotide labeled with a radioactive isotope (Agrawal, *et al.*, 1991; Cossum, *et al.*, 1993; Iverson, *et al.*, 1994). The most common isotopes are sulfur-35 and tritium (hydrogen-3). These can be made in-house, although they can also be obtained commercially. For a very simple study regarding circulatory lifetime and urine and fecal elimination, as little as 1 to 5 µCi per mouse will suffice, depending on the sensitivity of your equipment. More significant quantities will be needed to follow the degradation rate *in vivo* in the next stage of investigations.

The road to FDA approval from here is still long and difficult. However, you should now be well on your way and thinking about the second and third drugs in your pipeline.

### Final Words

Regardless of your intended use for antisense oligonucleotides, if you pay attention to those who have gone before, you stand a good chance of succeeding. This is especially true if you are developing an *in vitro* assay. If all else fails, there are companies in existence that are in the business of helping you develop your antisense assays. It may cost you more at first, but the expertise you purchase is usually well worth the price if your goals are commercial in nature. If you prefer to do it yourself, then go forward with the confidence of knowing your chances will be good in the long run and with the

knowledge that there are plenty of people out there willing to answer your questions.

## References

- Agrawal, K., and Riftina, F. (1979) *Nucleic Acids Research*, 6, 3009-3023.
- Agrawal, S., and Goodchild, J. (1987) *Tetrahedron Letters*, 28, 3539-3542.
- Agrawal, S., Temsamani, J. & Tang, J.Y. (1991) *Proc. National Acad. Sci.*, 88, 7595-7599
- Agrawal, S., Zhang, X., Lu, Z., Zhao, H., Tamburin, J.M., Yan, J., Cai, H., Diasio, R.B., Habus, I., Jiang, Z., Iyer, R.P., Yu, D. and Zhang, R. (1995) *Biochemical Pharmacology*, 50, 571-576.
- Blake, K., Murakami, A., Spitz, S.A., Glave, S.A., Reddy, M.P., Ts'o, P.O.P., & Miller, P.S. (1985) *Biochemistry*, 24, 6139-6145.
- Boggs, R.T., McGraw, K., Condon, T., Flourmoy, S., Villiet, P., Bennett, C. F., & Monia, B. P. (1997) *Antisense Nucleic Acid Drug Dev.*, 7, 461-471.
- Boziau, C., Dausse, E., Mishra, R., Duconge, F., & Toulme, J.-J. (1997) *Antisense Nucleic Acid Drug Dev.*, 7, 369-380
- Brown, D.A., Kang, S.H., Gryaznov, S.M., DeDionisio, L., Heidenreich, O., Sullivan, S., Xu, X., Nerenberg, M. I. (1994) *J. Biol. Chem.*, 269, 26801-5.
- Brysch, W., and Schlingensiepen, K. (1994) *Cellular and Molecular Neurobiology*, 14, 557-568.
- Capaccioli, S., Pasquale, G.D., Mini, E., Mazzei, T. and Quattrone, A. (1993) *Biochem. And Biophys. Res. Comm.*, 197, 818-825.
- Cohen, J.S. (1989) *TIBS*, 10, 435-437.
- Cossum, P.A., Sasmor, H., Dellinger, D., Truong, L., Cummins, L., Owens, S.R., Markham, P.M., Shea, J.P. and Crooke, S. (1993) *Journal of Pharm. and Exper. Therapeutics*, 267, 1181-1190.
- Dash, P., Lotan, I., Knapp, M., Kandel E.R. and Goelet, P. (1987) *Proc. Natl Acad. Sci. USA*, 84, 7896-7900.
- Dean, N.M., and McKay, R (1994) *Proc. Natl Acad. Sci. USA*, 91, 11762-11766.
- Desjardins, J.P., and Iverson, P.L. (1995) *J. of Pharmacology and Experimental Therapeutics*, 275, 1608-1613.
- Furdon, P.J., Domininski, Z., and Kole, R. (1989) *Nucleic Acids Research*, 17, 9193-9204.
- Giles, R.V., & Tidd, D.M. (1992) *Nucleic Acids Research*, 20, 763-770.
- Gryaznov, S., and Chen, J.K. (1994) *Journal of American Chemical Society*, 116, 3143-3144.
- Gura, T. (1995) *Science*, 70, 575-577.
- Hanvey, J.C., Peffer, N.J., Bisis, J.E., Thomson, S.A., Cadilla, R., Josey, J.A., Ricca, D.J., Hassman, C.F., Bonham, M.A., Au, K.G., Carter, S.G., Bruckenstein, D.A., Boyd, A.L., Noble, S.A., and Babis, L.E. (1992) *Science*, 258, 1481-1485.
- Hogrefe, H.H., Hogrefe, R.I., Walder, R.Y., Walder, J.A. (1990) *Journal of Biological Chemistry*, 265, 5561-5566.
- Iversen, P.L., Mata, J., Tracewell, W.G., & Zon, G. (1994) *Antisense Research and Development*, 4, 43-52
- Klinman, D.M. (1998) *Antisense Nucleic Acid Drug Dev.*, 8, 181-184.
- Krieg, A. M. (1998) *Handb. Exp. Pharmacol*, 131(Antisense Research and Application), 243-262.
- LaPlanche, L.A., James, T.L., Powell, C., Wilson, W.D., Uznanski, B., Stec, W.J., Summers, M.F., and Zon, G. (1986) *Nucleic Acids Research*, 14, 9081-9093.
- Lappalainen, K., Urtti, A., Soderling, E., Jaaskelainen, I., Syrjanen, K., and Syrjanen, S. (1994) *Biochemica and Biophysica Acta*, 201-208.
- Matzura, H., and Eckstein, F. (1968) *Eur. J. Biochem.*, 3, 448-452.
- Meteliev, V., Lisiewicz, J., and Agrawal, S. (1994) *Bioorg. Med. Chem. Lett.*, 4, 2929-34.
- Mignet, N., and Gryaznov, S.M. (1998) *Nucleic Acids Research*, 26, 431-438.
- Minshull, J., and Hunt, T. (1986) *Nucleic Acids Research*, 14, 6433-6451.
- Millan, C.L.B., Weeratna, R., Krieg, A. M., Siegrist, C., Davis, H.L. (1998) *Proc. Natl. Acad. Sci. U. S. A.*, 95, 15553-15558
- Miller, P.S. (1991) *Bio/Technology*, 9, 358-362.
- Monia, B.P., Lesnik, E. A., Gonzalez, C., Lima, W.F., McGee, D., Guinasso, C. J., Kawasaki, A. M., Cook, P. D., & Freier, S. M. (1993) *J. Biol. Chem.*, 268, 14514-22.
- Monia, B.P. (1997) *Ciba Found. Symp.*, 209 (Oligonucleotides as Therapeutic Agents), 107-123.
- Morvan, F., Sanghvi, Y., Perbost, M., Vasseur, J.-J., Bellon, L., (1996) *Journal of American Chemical Society*, 118, 255-256.
- Noonberg, Sarah B., Weiss, Tania L., Garovoy, Marvin R., Hunt, C. Anthony (1992) *Antisense Res. Dev.*, 2, 303-13.
- Quatrone, A., Pasquale, G.D., and Capaccioli, S. (1995) *Biochemica*, 1, 25-29.
- Peng Ho, S., Livanov, V., Zhang, W., Li, J., Leshner, T. (1998) *Mol. Brain Res.*, 62, 1-11.
- Ramasamy, R., Kanagaratnam, R., Misiura, K., Rebowski, G., Amerakoon, R., and Stec, W.J. (1996) *Biochem. and Biophys. Res. Comm.*, 218, 930-933.
- Reynolds, M.A., Hogrefe, R.I., Jaeger, J.A., Schwartz, D.A., Riley, T.A., Marvin, W.B., Daily, W.J., Vaghefi, M.M., Beck, T.A., Knowles, S.K., Klem, R.E. & Arnold, L.J. (1996) *Nucleic Acids Research*, 24, 4584-4591.
- Sasaki, M., Watanabe, M., Kawakubo, Y., Komiyama, M., Kobayashi, M., & Fujita, H. (1995) *Photomed., Photobiol.*, 17, 103-4
- Shoji, Y., Akhtar, S., Periasamy, A., Herman, B., & Juliano, R. L (1991) *Nucleic Acids Research*, 19, 5543-5550
- Sierakowaka, H., Sambade, M. J., Agrawal, S., & Kole, R. (1996) *Proc. Natl. Acad. Sci. U. S. A.*, 93, 12840-12844.
- Srinivasan, S. K., & Iversen, P. L. (1995) *Journal of Clinical Laboratory Analysis*, 9, 129-137.
- Stein, C.A., (1995) *Nature Medicine*, 1, 1119-1120.
- Stein, C.A., Subasinghe, C., Shinozuka, K., and Cohen, J.S. (1988) *Nucleic Acids Research*, 16, 3209-3221.
- Stephenson, M.L., and Zamecnik, P.C. (1978) *Proc. Natl Acad. Sci. USA*, 75, 1302-1306.
- Swayze, E.E., Bhat, B., Peoc'h, D., Sanghvi, Y. S. (1997) *Nucleosides Nucleotides*, 16, 971-972.
- Walder, R.Y., and Walder, J.A. (1988) *Proc. Natl Acad. Sci. USA*, 85, 5011-5015.
- Woolf, T.M., Melton, D.A., and Jennings, C.G.B. (1992) *Proc. Natl Acad. Sci. USA*, 89, 7305-7309.
- Zhou, W., and Agrawal, S. (1998) *Bioorg. Med. Chem. Lett.*, 8, 3269-3274.